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Report

Large-Scale Metagenomic-Based Study of Antibiotic Resistance in the Environment

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Summary

Antibiotic resistance, including multiresistance acquisition and dissemination by pathogens, is a critical healthcare issue threatening our management of infectious diseases [1–3]. Rapid accumulation of resistance phenotypes implies a reservoir of transferable antibiotic resistance gene determinants (ARGDs) selected in response to inhibition of antibiotic concentrations, as found in hospitals [1, 3–5]. Antibiotic resistance genes were found in environmental isolates, soil DNA [4–6], secluded caves [6, 7], and permafrost DNA [7, 8]. Antibiotics target essential and ubiquitous cell functions, and resistance is a common characteristic of environmental bacteria [8–11]. Environmental ARGDs are an abundant reservoir of potentially transferable resistance for pathogens [9–12]. Using metagenomic sequences, we show that ARGDs can be detected in all (n = 71) environments analyzed. Soil metagenomes had the most diverse pool of ARGDs. The most common types of resistances found in environmental metagenomes were efflux pumps and genes conferring resistance to vancomycin, tetracycline, or β-lactam antibiotics used in veterinary and human healthcare. Our study describes the diverse and abundant antibiotic resistance genes in nonclinical environments and shows that these genes are not randomly distributed among different environments (e.g., soil, oceans or human feces).

Results

A global environmental survey was performed with 71 environmental shotgun metagenomic DNA data sets obtained from public-repository websites. These data sets were derived from sequencing DNA extracted from various environmental samples (Environmental Shotgun Sequencing, EES) [12, 13]. Two different sequencing technologies were used to generate these data sets (Roche 454 and Sanger sequencing). These data sets equal a total of 8 Gb (equivalent to about 1,550 *Escherichia coli* genomes). Details concerning these data sets can be found in Table S1 available online. The environmental metagenomic reads from these data sets were compared to the Antibiotic Resistance Database (ARDB; http://ardb.cbcb.umd.edu), which contains 2,999 different amino acid sequences known to confer antibiotic resistance phenotypes to their host [13, 14]. Every metagenomic data set contained sequences with potential antibiotic resistance based on their similarity to the ARDB at the cutoff threshold used (i.e., BLASTx bit score >60 and alignment identity >35% [Table S4]; see the Supplemental Experimental Procedures for detailed experimental procedures). This threshold was chosen in order to minimize false positives when ARDB was used according to Looft et al. [14, 15]. Sequences matching the ARDB were then reannotated with a generalist database (NCBI_nr, release November 2013; SEED), which confirmed specificity of the antibiotic resistance annotation when SEED annotation was used (see Figures S2 and S3 for details). The SEED annotation system was designed to annotate 2,133 different functions at the subsystem level [15, 16], but it lacks the resolution for more-specific functions, such as antibiotic resistance. Therefore, 94% (n = 121,583) of the reads that were annotated as resistance genes on the basis of the ARDB were placed in the "not assigned" category in SEED (Figure S2). The same general trends were observed with both annotation procedures, but results obtained with ARDB annotations were more consistent regarding antibiotic-resistance specific annotations as expected for a dedicated database. This was essentially due to the use of a function-specific, curated, database. Results obtained with SEED using NCBI_nr BLAST results are available in Figures S2 and S3 and Table S5, and the ARDB annotations are described below.

Total Antibiotic Resistance Gene Determinant Abundance and Diversity in Each Environment

Relative antibiotic resistance gene determinant (ARGD) abundance ranged from 0.05% in chicken gut (metagenome accession number 4440284.3) to 5.6% in human feces (4440942.3) and differed significantly (at 95% confidence interval) among the different environmental data sets (Figure 1; see also Table S1). Data sets from environments with the highest proportion of ARGD-annotated sequence reads were the three human feces data sets from the Japan 29 Healthy Human Gut project (5.6% to 5.3% of all reads; Sanger sequencing), mouse gut (4.6% and 4.3% of reads; Sanger sequencing), and activated sludge (4.3% to 4.2% of reads; Sanger sequencing) (Figure 1). Human feces metagenomes obtained with Roche 454 pyrosequencing technology had less than 1% ARGD relative abundance. Sequencing technology can significantly influence annotation and needs to be taken into consideration when looking at metagenomic data. The longer reads obtained by Sanger were easier to annotate, thereby increasing their relative annotated read frequency, but such data sets have lower read counts compared to high-throughput next-generation sequencing technologies, which are harder to annotate due to their relatively shorter reads such as those obtained from Roche 454 [1, 3, 16, 17]. Still, Sanger-sequenced human feces data sets contained relatively more ARGD-annotated reads than did environmental data sets from oceans or farm soil also obtained with Sanger. All three Sanger-sequenced data sets had higher relative numbers of ARGD-annotated reads than cow gut, chicken gut, and six ocean data sets, which were obtained by Roche 454. These Roche 454-sequenced data sets are also those with the shortest mean read length (about 100 bp) among the studied metagenomes. The ARGD relative diversity found in each environment was estimated

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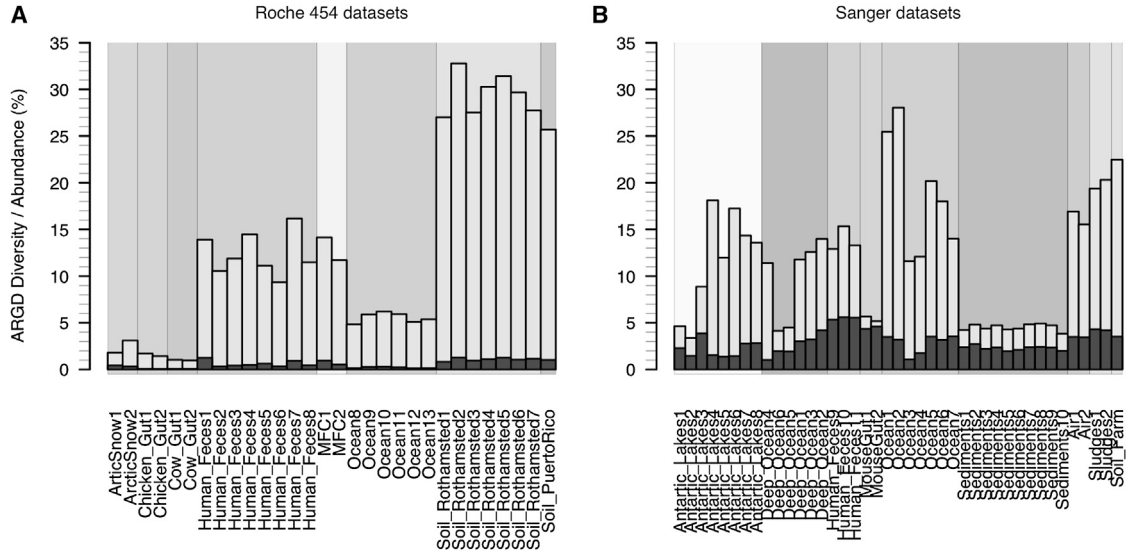

Figure 1. Relative Diversity and Abundance of ARGDs between Metagenomes

Bar plot showing the relative abundance of reads annotated as ARGDs (gray bars), expressed as the number of ARGD hits divided by the total number of reads in the data set, and ARGD diversity (white bars), expressed as the percentage of the 2,999 unique sequences in the ARDB found in the Roche 454 (A) and Sanger (B) data sets. Shaded boxes indicate a different environmental origin with, from left to right, Antarctic lakes, Arctic snow, chicken gut, cow gut, deep oceans, human feces, microbial fuel cell, mouse gut, ocean, halophile sediment, activated sludge, and soil. Black dots indicate data sets obtained by Sanger sequencing method; others were obtained by Roche 454 sequencing.

as a percentage of the 2,999 different sequences from the ARDB detected in the corresponding sequence data sets (Figure 1). Overall, the percentage of different ARGD sequences detected ranged from 0.97% in cow gut (4441680.3) to 32.78% in Rothamsted soil E41. Our results showed that soil had the highest level of ARGD diversity, with an average of 28.8% ± 3.14% of the database sequences found in the soil data sets (a maximum of 32.8% in Rothamsted E41 and a minimum of 22.5% in Waseca Farm Soil) (Figure 1). Data sets from cow rumen, chicken cecum, and arctic snow had the lowest number of different ARGD sequences, with only 1.3% of the ARDB database sequences detected in the cow rumen, chicken cecum, and arctic snow metagenomic data sets. Metagenomic data sets from human feces from different origins and sequencing technologies had an average of 12.7% ± 2.1% of the different ARDB database sequences (a maximum of 16.17% in Twin Study 4440616.3 and a minimum 9.3% in Twin Study 4440614.3). This observation is consistent with the general trend of a reduced diversity level of antibiotic resistance genes in nonsoil environments compared to soil (Figure 1).

Most Prevalent Resistance Types in the Environment

The *macab* and *bcr* efflux pumps accounted for 18.9% and 8.5% of the total ARGD hits in all environments, respectively (Table 1; details available in Table S2 and Figure S1). Most ARGD-annotated reads detected in metagenomic data sets using our stringent annotation cutoff were related to various types of efflux-pump components: membrane fusion protein from resistance-nodulation cell division efflux pumps (RND) and ATP-binding cassette (ABC) domains related to bacitracin efflux, with 27.9% and 20.8% of the total hits of ARGD annotated sequences in all metagenomes, respectively (Table 1; see also Table S2). Tetracycline ribosomal protection protein-encoding genes (*tet_rpp*, 5.7%) and vancomycin resistance-related genes (*van a*, 3.6%; *van b*, 2.9%; *van d*, 3.5%; *van e*, 2.3%; *van g*, 2.8%; and *van c*, 1.8%) were also found. Penicillin binding protein (*Pbp*) accounted for 5.4% of annotated reads. β-lactamases were less frequently detected (class A β-lactamases, *bla_a*, 3.4%; class B β-lactamases, *bla_b*, 0.2%; class C β-lactamases, *bla_c*, 0.42%; and class D β-lactamases, *bla_d*, 0.04%; Table S2).

ARGD Distribution Shows Distinct Environmental Clusters

In order to overcome the bias related to the sequencing method for ecosystem comparison, we carried out an additional ARGD distribution analysis, based on a clustering method, on a restricted subset of metagenomic sequences obtained by Roche 454 pyrosequencing. Sanger data sets were insufficiently homogeneous and therefore were discarded from the analysis. The dendrogram obtained was plotted via Ward hierarchical classification of the data sets Mountford index (see the Supplemental Experimental Procedures). The Mountford index reflects similarities between samples based on ARGD sequence presence or absence for each metagenome data set (i.e., ARGD beta diversity is defined as a function of alpha and gamma diversities). The different data sets used in the analysis were clustered in three different groups that matched their environmental origin: ocean group, soil group, and human feces group (Figure 2). Soil data sets were similar regardless of the soil's geographical origin or usage (e.g., a prairie soil in England or a forest soil in Puerto Rico), whereas two separate clusters were identified for ocean data sets. Open-ocean data sets grouped separately from coastal-ocean data sets. The Venn diagram of resistance class level annotations showed a common set of antibiotic resistance genes shared between all three environmental clusters (Figure 3; see also Table S3). Among all different ARGD types (n = 94), the majority (n = 50) were common to all environments, and less than 10% (n = 6) were not found anywhere. The six types of ARGD not detected in any cluster were *dfrb* (group B trimethoprim-resistant dihydrofolate reductase),

Table 1. Details for the 50 Most Prevalent Resistance Classes Found in All Metagenomes

Resistance Class	Mechanism of Resistance	Antibiotic Specificity
mexef, ceo, mexvw, acr, mexhi, mexcd, mexab, mdtnop, amr, adeabc, smeabc, smedef, mdtef, mexxy, mdtk	RND class transporter	multidrug resistance efflux
macab	RND class transporter: macrolide	multidrug resistance efflux
bcr, bcr_mfs	ABC class transporter system: bacitracin	multidrug resistance efflux
mls_abc	ABC class transporter: macrolide	multidrug resistance efflux
mls_mfs, mls_hdr	MFS class transporter: macrolide	multidrug resistance efflux
cml	MFS class transporter: chloramphenicol	multidrug resistance efflux
rosab	potassium antiporter system	multidrug resistance efflux
mepa, norm	MATE transporter	multidrug resistance efflux
tcma, mdr, qac	MFS transporter	multidrug resistance efflux
vana, vanb, vanc, vand, vane, vang	vancomycin resistance operon genes (vanH, vanS, vanR, vanX, and vanY) for each vancomycin resistance operon: VanA, VanB, VanC, VanD, VanE, and VanG types	vancomycin
tet_rpp	tetracyline ribosomal protection protein	tetracycline
tet_efflux	tetracyline-specific efflux pump	tetracycline
tet_flavo	flavoproteins resistance to tetracyline	tetracycline
bla_a, bla_b	class A and class B β-lactamases	β-lactams
pbp	penicillin-binding protein	β-lactams
baca	bacitracin resistance	bacitracin
cata	chloramphenicol acetyltransferase	chloramphenicol
ksga	kasugamycin resistance	kasugamycin
arna	polymixin resistance	polymixin
pur8	puromycin resistance	puromycin
vat	virginiamycin resistance	streptogramin
sul	sulfonamide resistance	sulfonamide
dfra	trimethoprim resistance	trimethoprim

Resistance classes are groups of genes implicated in the same resistance phenotype as defined in the ARDB (http://ardb.cbcb.umd.edu). The most prevalent resistance classes found in our analysis are MDR efflux pumps, vancomycin resistance, tetracycline resistance, β-lactams resistance, and resistance to other antibiotic molecules: polymixin, bacitracin, sulfonamide, kasugamycin, puromycin, trimethoprim, streptogramin, and chloramphenicol. See also Table S2 and Figure S1 for resistance mechanisms involved and antibiotic molecule targets for each group of the 50 resistance classes found, as well as their respective abundance.

fusb (fusaric acid resistance), *mph* (macrolide phosphotransferase), *rif* (viral rifampin resistance), *vph* (viomycin phosphotransferase), and tet_other (tetracycline resistance genes that are not efflux pumps or ribosomal binding proteins). The ocean and human feces clusters did not share any ARGD type. Multidrug efflux pump class *lmrp* was found only in the human feces cluster. ARGDs shared between soil and ocean clusters (n = 3) were genes conferring resistance to fosfomycin, puromycin, and quinolones. A large subset (n = 24) of ARGD classes was shared between human feces and soil clusters. This subset includes all classes of β-lactamases, resistances to aminoglycoside through antibiotic molecules modification (phosphotransferases, acetyltransferases, or nucleotidyltransferases), various multidrug efflux pumps, and flavoproteins conferring resistance to tetracycline.

Discussion

The objective of our study was to evaluate the quantity and diversity of antibiotic resistance genes from different environments using the metagenomic data sets from various biomes (soils, ocean, human gut, etc.). Metagenomic sequence reads were annotated as ARGD if their sequence matches a set of known antibiotic resistance genes. Our results show that soil environments have the largest apparent diversity of ARGDs, whereas gut-associated microbiota have a higher proportion of ARGD-annotated reads, which may be due to exposure to antibiotic molecules that increased the overall antibiotic resistance of the commensal flora [1, 3, 17, 18].

Efflux pumps dominated the best-hit-only annotation (i.e., not removing best hits below a bit score and percent identity threshold of above 60% and 35%, respectively). Antibiotic-specific efflux pumps share homologies at the amino acid level with other pump genes and share functional domains with every efflux pump protein (e.g., membrane-fusion protein [MFP] domain). Efflux pumps are present in both antibiotic-susceptible and antibiotic-resistant bacteria and can be poor markers of resistance phenotype. Efflux-pump-related resistance is often conferred by point mutations in their regulatory genes [5, 18, 19]. However, in our analysis, most detected efflux pump reads were related to efflux of antibiotic or toxic compounds even when re-examined with NCBI_nr (Figure S3). This implied that the antibiotic resistance hit was not superseded by a hit with a generalist database. Some ARGD protein families with many different variants, such as β-lactamases or efflux pumps, were more abundantly referenced in our database, and that might have increased detection potential compared to ARGDs with few representatives in the ARDB. ARGDs, such as vancomycin resistance operons, β-lactamases, or tetracycline ribosomal protection proteins, usually confer the resistance phenotype directly when translated. This makes these ARGD-annotated reads better markers of potential antibiotic resistance phenotypes. ARGDs were systematically detected in every environment screened despite all the described limitations. ARGDs are probably even more abundant in all of the ecosystems than what has been observed with sequence-based analysis. Thus, we believe that our method retrieved many bona fide ARGDs from the environmental resistome.

Metagenomes obtained with the same technology form three distinct environmental groups (ocean, soil, and human feces) on the basis of presence or absence of annotated

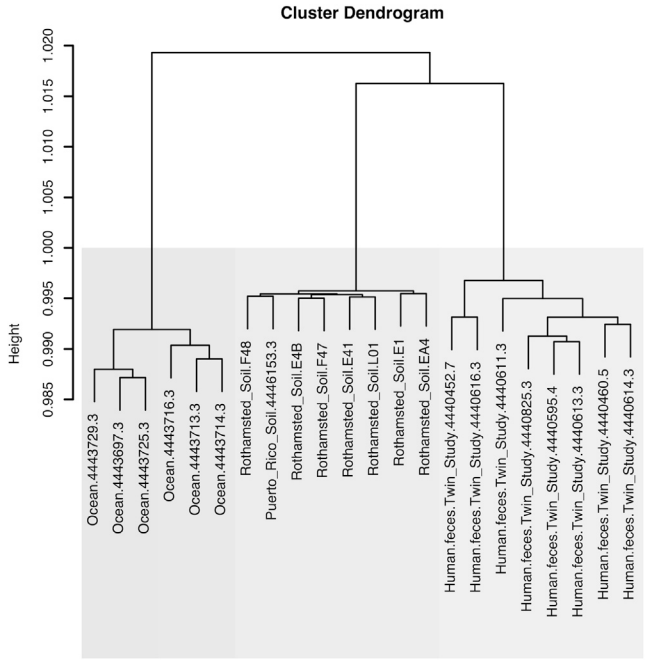

Figure 2. Hierarchical Clustering Based on Mountford Diversity Index of ARGD among Soil, Ocean, and Human Feces Roche 454 Metagenomic Data Sets

Mountford index has been computed from the distribution of ARGD hits in each metagenomic data set considering each different ARGD as a different species. Hierarchical clustering based on ward method has been performed on Mountford index matrix. Environmental clusters are highlighted with colors as follows: dark blue, open ocean; light blue, coastal ocean; brown, soil; and red, human feces.

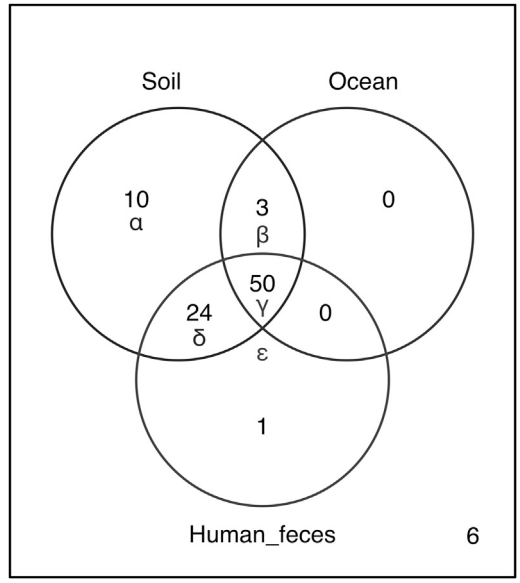

Figure 3. Common ARGD Class between Four Different Environmental Clusters Based on Roche 454 Data Sets

Numbers inside each circle intersection indicate the number of common resistance classes between environmental data sets. Only in soil (α): mls_hdr, pmra, qacab, qnr, tmr, ble, *bmr*, *emea*, *ere*, and *fush*. Common to soil and ocean only β: *fos* (fosfomycin resistance), *pac* (puromycin resistance), and *mfpa* (quinolones resistance). Common to soil, ocean, and human feces (γ): *acr*, *adeabc*, *amr*, *macab*, *mdtef*, *mdtk*, *mdtm*, *mdtnop*, mexab, mexcd, mexef, mexhi, mexvw, mexxy, smeabc, smedef (RND efflux pumps), *arna* (polymixin resistance), *baca*, *bcr*, *bcr_mfs* (bacitracin resistance), *catb*, *ceo*, *cml* (chloramphenicol resistance), *dfra* (group A trimethoprim-resistant dihydrofolate reductase), *emrd, emre*, *mdr*, *nora*, *norm*, *rosab*, *tcma* (MFS transporters), *erm*, *lsa* (macrolide resistance), *ksga* (kasugamycin resistance), *meca*, *pbp* (β-lactams resistance), mls_abc (ABC family transporter), *pur8* (puromycin resistance), *qac* (quaternary ammonium compound efflux pump), *sul* (sulfonamide-resistant dihydropteroate synthase), *tet_efflux*, *tet_xprt* (tetracycline efflux pumps), *tet_rpp* (tetracycline ribosomal protection protein), *vana*, *vanb*, *vanc*, *vand*, *vane*, *vang* (vancomycin resistance), and *vat* (virginiamycin A acteyltranferase). Common to soil and human feces (δ): *aac*, *ant*, *aph* (aminoglycoside transferases), *bla_a*, *bla_b*, *bla_c*, *bla_d* (β-lactamases), *blt*, *cata* (chloramphenicol acetyltransferase), *lnu* (lincomycin nucleotidyltransferase), *mdfa, mdtg, mdth, mdtl, mepa, mls_mfs, ykk* (Multidrug efflux pump), *mecr1* (methicillin resistance), *sta*, *str* (streptomycin resistance), *tet_flavo*, *tet_mod* (resistance to tetracycline), *vanz* (vancomycin resistance), and *tsnr* (thiosptrepton resistance). Only in human feces (ε): *lmrp* (MFS transporter). Not detected (6): *dfrb* (group B trimethoprim-resistant dihydrofolate reductase), *fusb* (fusaric acid resistance), *mph* (macrolide phosphotransferase), *rif* (viral rifampin resistance), *vph* (viomycin phosphotransferase), and tet_other (tetracycline resistance genes that are not efflux pumps or ribosomal binding proteins). See also Table S3.

ARGD sequences (Figure 2). While many ARGDs were shared between all environments (Figure 3), soil and human feces shared more (24 out of 94) ARGD classes (i.e., groups of sequences with shared antibiotic resistance-conferring mechanisms as defined in the ARDB [13]) than any other pairs of ecosystems. This heightened degree of shared ARGDs between soil and human feces data sets has been previously explored by functional metagenomics and led to the conclusion that gene flow between these environments is likely [4, 5, 19]. Soil is probably the major source of antibiotic-producing soil microorganisms (e.g., *Streptomyces chrysogenum* producing penicillin), and these microorganisms live in apparently the most diverse environment in terms of ARGDs (Figure 1). Considering this diversity, the environment and, more specifically, the soil reservoir of antibiotic resistance genes could be major contributors to antibiotic resistance mechanism diversification in pathogen populations by horizontal gene transfer [1].

The potential ecological pressure that low natural concentrations of antibiotics might exert has important implications concerning the role of antibiotics molecules and ARGDs in bacterial ecology. In environments without anthropogenic amendment of antibiotic molecules, such as the Rothamsted soil or the Alaskan permafrost, antibiotic molecule concentrations were not detected, yet functional antibiotic resistance genes were always detected [4, 5, 20, 21]. In addition to their antibacterial activity, antibiotic molecules have been found to act as signal molecules between bacterial cells [8, 20–24], and many antibiotic resistance genes are themselves involved in diverse bacterial physiological functions [8, 22–27]. Their possible multifunctional role and their low fitness costs (and eventual fitness benefits independent of antibiotic resistance [25]) might explain the prevalence of most ARGDs in the environment. Indeed, maintenance in their host may not require a constant selective pressure, making environmental bacteria a persistent source of ARGDs for pathogens [25–27]. Human feces data sets showed that antibiotic resistance genes were present in human-gut-resident bacteria, with as much as 5.6% ARGD-annotated reads. In human gut, other mechanisms of antibiotic resistance gene selection besides inhibition resistance could be involved. The most abundant ARGD-annotated reads detected in human data sets were vancomycin

resistance genes, even though vancomycin is a strongly regulated and rarely used "last resort" antibiotic in medicine. In contrast, amoxicillin-related resistance genes (β-lactamases) were much less abundant in human feces, although this drug is commonly used.

The ecological role of every ARGD in the environment has yet to be elucidated, but some ARGDs have been shown to play roles, such as intercellular signaling, other than antibiotic protection for their original (i.e., not clinical) saprophyte host [2, 25–27]. Antibiotic molecules and antibiotic resistance are an inherent part of common bacterial ecophysiology in the environment. This ARGD resource is selected in pathogens when they acquire this trait and enhance their fitness in the case of antibiotherapy. Yet, not all common pathogens are resistant to antibiotics. With the efficiency of bacteria to adapt to the antibiotic challenge via mutations, DNA recombination, plasmid- and integron-mediated gene transfer, they are outpacing the health industry in terms of development and innovation. Further studies on the mechanism of antibiotic resistance dispersion (e.g., the role of mobile genetic elements) are critical for enhancing antibiotic clinical lifetimes. Antibiotic resistance genes in the environment (and clinical antibiotic resistance) are not likely to disappear in the near future, although selection mechanisms seem to limit dissemination of antibiotic resistance. Deciphering these mechanisms may provide the tools for enhancing the long-term efficiency of currently available and future antibiotics.

Supplemental Information

Supplemental Information includes three figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.03.036.

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References

1. Ochman, H., Lawrence, J.G., and Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. Nature *405*, 299–304.
2. Cantón, R., and Morosini, M.-I. (2011). Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol. Rev. *35*, 977–991.
3. Lawrence, J.G., and Roth, J.R. (1996). Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics *143*, 1843–1860.
4. Allen, H.K., Moe, L.A., Rodbumrer, J., Gaarder, A., and Handelsman, J. (2009). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. ISME J. *3*, 243–251.
5. Demanèche, S., Sanguin, H., Poté, J., Navarro, E., Bernillon, D., Mavingui, P., Wildi, W., Vogel, T.M., and Simonet, P. (2008). Antibiotic-resistant soil bacteria in transgenic plant fields. Proc. Natl. Acad. Sci. USA *105*, 3957–3962.
6. Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A., and Wright, G.D. (2012). Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE *7*, e34953.
7. D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., et al. (2011). Antibiotic resistance is ancient. Nature *477*, 457–461.
8. Martinez, J.L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J.F., Martínez-Solano, L., and Sánchez, M.B. (2009). A global view of antibiotic resistance. FEMS Microbiol. Rev. *33*, 44–65.
9. Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R., and Philippon, A. (2002). Beta-lactamases of Kluyvera ascorbata, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother. *46*, 3045–3049.
10. Toleman, M.A., Spencer, J., Jones, L., and Walsh, T.R. (2012). blaNDM-1 is a chimera likely constructed in Acinetobacter baumannii. Antimicrob. Agents Chemother. *56*, 2773–2776.
11. Aminov, R.I., and Mackie, R.I. (2007). Evolution and ecology of antibiotic resistance genes. FEMS Microbiol. Lett. *271*, 147–161.
12. Eisen, J.A. (2007). Environmental shotgun sequencing: its potential and challenges for studying the hidden world of microbes. PLoS Biol. *5*, e82.
13. Liu, B., and Pop, M. (2009). ARDB—Antibiotic Resistance Genes Database. Nucleic Acids Res. *37* (Database issue), D443–D447.
14. Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. USA *109*, 1691–1696.
15. Overbeek, R.A., Begley, T., Butler, R.M., Choudhuri, J.V., Chuang, H.-Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T., Edwards, R.A., et al. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. *33*, 5691–5702.
16. Wommack, K.E., Bhavsar, J., and Ravel, J. (2008). Metagenomics: read length matters. Appl. Environ. Microbiol. *74*, 1453–1463.
17. Forslund, K., Sunagawa, S., Kultima, J.R., Mende, D.R., Arumugam, M., Typas, A., and Bork, P. (2013). Country-specific antibiotic use practices impact the human gut resistome. Genome Res. *23*, 1163–1169.
18. Piddock, L.J.V. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin. Microbiol. Rev. *19*, 382–402.
19. Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., and Dantas, G. (2012). The shared antibiotic resistome of soil bacteria and human pathogens. Science *337*, 1107–1111.
20. Aminov, R.I. (2009). The role of antibiotics and antibiotic resistance in nature. Environ. Microbiol. *11*, 2970–2988.
21. Fajardo, A., and Martínez, J.L. (2008). Antibiotics as signals that trigger specific bacterial responses. Curr. Opin. Microbiol. *11*, 161–167.
22. Wellington, E.M.H., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infect. Dis. *13*, 155–165.
23. Alonso, A., Sánchez, P., and Martínez, J.L. (2001). Environmental selection of antibiotic resistance genes. Environ. Microbiol. *3*, 1–9.
24. Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. *8*, 251–259.
25. Andersson, D.I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. *8*, 260–271.
26. Andersson, D.I., and Levin, B.R. (1999). The biological cost of antibiotic resistance. Curr. Opin. Microbiol. *2*, 489–493.
27. Hall, A.R., Iles, J.C., and MacLean, R.C. (2011). The fitness cost of rifampicin resistance in Pseudomonas aeruginosa depends on demand for RNA polymerase. Genetics *187*, 817–822.